

STIC-ILL

QH 506.MC

**From:** Hunt, Jennifer  
**Sent:** Tuesday, July 03, 2001 5:25 PM  
**To:** STIC-ILL  
**Subject:** Ref for 09/234,208

Please send me the following references ASAP.

Yamamoto et al., Nature 319: 230-234 1986

Scott et al., Mol. Cell Biology, 13:2247-2257, 1993

Petch et al., Mol. Cell Biology, 10:2973-2982, 1990

Basu et al., Mol. Cell Biology, 9: 671-677 1989

Flickinger et al., Mol. Cell Biology, 12: 883-893 1992

Thanks,

Jennifer Hunt  
Patent Examiner, Art Unit 1642  
CM1-8D06  
(703)308-7548

## A Truncated Intracellular HER2/*neu* Receptor Produced by Alternative RNA Processing Affects Growth of Human Carcinoma Cells

GARY K. SCOTT,<sup>1</sup> ROBERT ROBLES,<sup>1</sup> JOHN W. PARK,<sup>1,2</sup> PAMELA A. MONTGOMERY,<sup>1</sup> JANICE DANIEL,<sup>1</sup> WILLIAM E. HOLMES,<sup>2</sup> JAMES LEE,<sup>2</sup> GILBERT A. KELLER,<sup>2</sup> WEN-LU LI,<sup>2</sup> BRIAN M. FENDLY,<sup>2</sup> WILLIAM I. WOOD,<sup>2</sup> H. MICHAEL SHEPARD,<sup>2</sup> AND CHRISTOPHER C. BENZ<sup>1\*</sup>

*Cancer Research Institute, University of California, San Francisco, California 94143-0128,<sup>1</sup>  
and Genentech, Inc., South San Francisco, California 94080<sup>2</sup>*

Received 17 September 1992/Returned for modification 23 October 1992/Accepted 30 December 1992

Cloned sequences encoding a truncated form of the HER2 receptor were obtained from cDNA libraries derived from two HER2-overexpressing human breast cancer cell lines, BT-474 and SK-BR-3. The 5' 2.1 kb of the encoded transcript is identical to that of full-length 4.6-kb HER2 transcript and would be expected to produce a secreted form of HER2 receptor containing only the extracellular ligand binding domain (ECD). The 3' end of the truncated transcript diverges 61 nucleotides before the receptor's transmembrane region, reads through a consensus splice donor site containing an in-frame stop codon, and contains a poly(A) addition site, suggesting that the truncated transcript arises by alternative RNA processing. S1 nuclease protection assays show a 40-fold variation in the abundance of the truncated 2.3-kb transcript relative to full-length 4.6-kb transcript in a panel of eight HER2-expressing tumor cell lines of gastric, ovarian, and breast cancer origin. Expression of this truncated transcript in COS-1 cells produces both secreted and intracellular forms of HER2 ECD; however, immunofluorescent labeling of HER2 ECD protein in MKN7 tumor cells that natively overexpress the 2.3-kb transcript suggests that transcriptionally generated HER2 ECD is concentrated within the perinuclear cytoplasm. Metabolic labeling and endoglycosidase studies suggest that this HER2 ECD (100 kDa) undergoes differential trafficking between the endoplasmic reticulum and Golgi compartments compared with full-length (185-kDa) HER2 receptor. Transfection studies indicate that excess production of HER2 ECD in human tumor cells overexpressing full-length HER2 receptor can result in resistance to the growth-inhibiting effects of anti-HER2 monoclonal antibodies such as muMab4D5. These findings demonstrate alternative processing of the HER2 transcript and implicate a potentially important growth regulatory role for intracellularly sequestered HER2 ECD in HER2-amplified human tumors.

The HER2 (*neu*, *c-erbB-2*) proto-oncogene encodes a 185-kDa transmembrane protein tyrosine kinase receptor with extensive homology to the epidermal growth factor (EGF) receptor, HER1 (4, 6, 20, 36). The need to understand the role of the HER2 receptor, and its growth-regulating ligand(s) (15, 27, 32), is underscored by the prevalence of HER2 DNA amplification in a variety of human epithelial cancers, including those of ovarian, gastrointestinal, and mammary origin, in which the overexpressed receptor contributes to aggressive tumor growth and reduced patient survival (13, 37). Moreover, newly developed anti-HER2 monoclonal antibodies that bind with high affinity to the receptor's extracellular domain (ECD) result in both in vitro and in vivo growth inhibition of HER2-overexpressing tumors (10, 14, 17, 31, 38). One of these murine monoclonal antibodies, muMab4D5, is now involved in clinical testing for the treatment of patients with HER2-overexpressing breast and ovarian cancers (31).

Tumors and cell lines overexpressing transmembrane growth factor receptors often release truncated receptor ECD. The truncated forms of these receptors may arise by proteolytic cleavage of the full-length receptor, as for colony-stimulating factor 1 and interleukin-2 receptors (7, 26, 34), and/or by alternate splicing of receptor transcript into a form that eliminates the transmembrane and cytoplasmic

domains, as has been shown for interleukin-4, Fc, and EGF receptors (29, 30, 33). Soluble HER2 ECD is released from HER2-overexpressing tumor cells in vitro and in vivo (1, 24, 41), and the mechanism for this is thought to involve surface proteolysis of the 185-kDa receptor (41). Largely unexplored are the potential physiological effects of truncated growth factor receptors, which might involve extracellular competition for cognate ligand (5) or intracellular dominant-negative suppression of receptor function by heterodimer formation (11, 20).

We report here the isolation of clones encoding a truncated ECD form of the HER2 receptor from cDNA libraries prepared from two HER2-overexpressing human breast cancer cell lines, BT-474 and SK-BR-3. The truncated HER2 ECD transcript appears to be produced by an alternative RNA processing mechanism in which an exon extends through a splice site utilized by the full-length transcript, providing an in-frame stop codon and an alternate poly(A) addition site. The 2.3-kb truncated HER2 transcript is variably expressed in a panel of human epithelial cancer cell lines and produces intracellular HER2 ECD protein of about 100 kDa. Transfection studies suggest that excess production and intracellular retention of 100-kDa HER2 ECD in tumor cells overexpressing 185-kDa HER2 receptor results in resistance to the growth-inhibiting effects of the anti-HER2 monoclonal antibody muMab4D5. These findings demonstrate a potentially important growth regulatory role for alternative processing of the HER2 transcript and the

\* Corresponding author.

intracellular production of HER2 ECD in HER2-overexpressing human tumors.

## MATERIALS AND METHODS

**Cell lines.** Human breast carcinoma lines SK-BR-3, BT-474, MCF-7, MDA-MB-453, MDA-MB-468, and T47D and the ovarian carcinoma cells SK-OV-3 were obtained from the American Type Culture Collection (Rockville, Md.) and maintained as recommended. The human gastric carcinoma cell line MKN7 was provided by T. Yamamoto (40). Cell growth assays in the presence of the anti-HER2 murine monoclonal antibody muMab4D5 were performed as previously described (35).

**cDNA cloning and sequencing.** cDNA libraries were prepared from poly(A) RNA derived from the HER2-overexpressing human breast cancer cell lines BT-474 and SK-BR-3. Reverse transcription of RNA into cDNA was performed as described by Gubler and Hoffmann (12). Blunt-ended cDNA from BT-474 poly(A) RNA was ligated to hemiphosphorylated *Eco*RI adaptors (Promega, Madison, Wis.) and cloned into the  $\lambda$ gt10 phage vector system. Blunt-ended cDNA from SK-BR-3 was ligated to *Bst*XI adaptors and cloned into pRK5, an expression plasmid using the cytomegalovirus promoter/enhancer with simian virus 40 (SV40) termination and polyadenylation signals located downstream of the inserted cDNA. Clones from the BT-474  $\lambda$ gt10 library which hybridized to the 5' HER2 cDNA probe  $\lambda$ MAC14-1 (21), but did not hybridize to a 3' HER2 cDNA probe (800-bp *Pst*I-*Eco*RI fragment beginning 45 bp downstream from the transmembrane coding domain), were selected for further analysis. From the SK-BR-3 pRK5 expression library, pools of cDNA clones were transfected by using DEAE-dextran into COS-1 cells. Conditioned media were collected, concentrated 40-fold in 10-kDa-cutoff Microsep filters (Filtron Technology Corp., Northborough, Mass.), and screened for inhibition of SK-BR-3 cell binding to iodinated muMab4D5, which recognizes only the extracellular domain of HER2 (17). From one pool that produced a marked reduction in cell surface antibody binding, successive rounds of serial dilution resulted in the isolation of a single clone, pW597.3A, whose secreted protein product repressed muMab4D5 binding to the HER2 receptor on SK-BR-3 cells. *Eco*RI inserts from cDNA clones were subcloned into pBSKS<sup>-</sup> (Stratagene, La Jolla, Calif.) and sequenced by the dideoxy technique (Sequenase; U.S. Biochemical, Cleveland, Ohio), with either single-stranded phage or double-stranded plasmid (prepared by Magic Mini Preps; Promega) as the template and using insert flanking primers (M13 forward and reverse primers) and primers prepared from the HER2 cDNA sequence (6).

**Polymerase chain reaction (PCR) analysis.** DNA samples (0.1  $\mu$ g of BT-474 DNA and 1  $\mu$ g of human placenta DNA) were combined with 50 pmol of both a 3' primer (5'-CCTTTTATAGTAAGAGCCCC-3') designed to prime 78 bp 3' of the divergence and a 5' primer (5'-TGAGGAGGGCG CATGCCA-3') designed to prime 45 bp 5' of the divergence in a 50- $\mu$ l reaction volume containing 10 mM Tris (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.001% gelatin, 200  $\mu$ M each deoxynucleoside triphosphate and 1 U of AmpliTaq (Perkin-Elmer/Cetus), and sample reactions were subjected to 34 thermal cycles (1 min at 94°C, 1.5 min at 48°C, and 1 min at 72°C). Aliquots (7  $\mu$ l) were then electrophoresed through a 12% polyacrylamide gel in 1 $\times$  TBE (0.09 M Tris, 0.09 M boric acid, 0.002 M EDTA). After electrophoresis, the gel was treated with 50 mM NaOH for 10 min (8), neutralized in

1 $\times$  TBE, and then electroblotted to Hybond (Amersham, Arlington Heights, Ill.) in 1 $\times$  TBE. The DNA was UV cross-linked to the filter and probed with the 3'-terminal sequences from the truncated HER2 cDNA. Final filter washing was at 68°C in 0.2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1% sodium dodecyl sulfate (SDS).

**RNA analysis by Northern (RNA) blotting and S1 nuclease protection.** Total RNA was prepared by the guanidinium isothiocyanate-CsCl step-gradient technique and Northern blotted as previously described (25, 36). Ten micrograms of total RNA was electrophoresed through 1.0% agarose formaldehyde gels, transferred to a Hybond membrane (Amersham), and UV cross-linked, and the filters were then probed in 50% formamide-5 $\times$  SSC-1% SDS at 42°C. Filters were given final washes at 68°C in 0.2 $\times$  SSC-1% SDS. Preparation of single-stranded DNA probes for S1 nuclease protection assay were generated by using a variation of the procedure based on priming single-stranded phage DNA. A 196-bp *Ppu*MI-*Xho*II fragment from pW597.3A, in which the *Ppu*MI site was filled by Klenow enzyme prior to *Xho*II digestion, was cloned into *Hinc*II-*Bam*HI-digested pTZ18R (Pharmacia) to produce pTZ18RW5; this fragment was then digested with *Hind*III, purified, and subjected to exonuclease III digestion to uncover the reverse priming site 281 bp downstream of the *Hind*III cut site. To 0.1  $\mu$ g of exonuclease III-digested pTZ18RW5, 1 pmol of M13 reverse primer (17-mer; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was annealed (50°C, 30 min) and then extended with Klenow enzyme, using [ $\alpha$ -<sup>32</sup>P]dCTP (800 Ci/mmol) as the radiolabeled nucleotide. The reaction was then boiled in an equal volume of formamide loading buffer (95% formamide, 1 mM EDTA [pH 8.0]) and electrophoresed on a 5% polyacrylamide-8 M urea gel. The 281-nucleotide (nt) single-stranded DNA band was excised (other radiolabeled bands were >2.7 kb) and purified, and approximately 5  $\times$  10<sup>4</sup> cpm of the single-stranded DNA probe was added to 30  $\mu$ g of sample RNA. The sample mixture was precipitated with ethanol, resuspended in hybridization buffer [40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, 1 mM EDTA, 80% formamide], and incubated for 15 h at 58°C; 300  $\mu$ l of S1 nuclease buffer (50 mM sodium acetate [pH 4.6], 0.3 M NaCl, 4.5 mM ZnSO<sub>4</sub>) containing 200 U of S1 enzyme per ml was added to each hybridization reaction, and the mixture was incubated at 23°C for 30 min. The S1 digestion was terminated by phenol-chloroform extraction, ethanol precipitated in the presence of 3  $\mu$ g of sheared salmon sperm DNA, resuspended in 10  $\mu$ l of formamide loading buffer, boiled, and electrophoresed on a 5% polyacrylamide-8 M urea gel. The gel was then dried and autoradiographed, and bands (281, 181, and 125 nt) were quantitated by scanning densitometry to determine the ratio of full-length to truncated HER2 transcript (125-nt band/181-nt band). Because of the added length and resultant signal intensity of the protected 181-nt fragment, these densitometry ratios were corrected by a factor of 1.6 to calculate the true HER2 transcript ratios.

**Cell transfection, protein expression, and immunoblotting.** Expression plasmid pW597.3A was transiently transfected into COS-1 cells by DEAE-dextran; 3 days after COS-1 transfection, the medium was replaced with serum-free medium and incubation continued for another 24 h. The conditioned medium was then collected and concentrated 35-fold by using 10-kDa-cutoff Microsep filters. Cell lysates of the transfected COS-1 cells (C-pW597.3A) were prepared by rinsing the cells in 1 $\times$  phosphate-buffered saline (PBS)

and then harvesting the cells in cell lysis buffer (20 M Tris [pH 8.0], 137 mM NaCl, 0.1% SDS, 1% Nonidet P-40). MDA-MB-453, SK-BR-3, and BT-474 cultures were permanently transfected with pW597.3A by calcium phosphate precipitation (25). MDA-MB-453 and SK-BR-3 cells were cotransfected with a control (selectable) expression plasmid containing a neomycin phosphotransferase gene (*neo*) under SV40 promoter control (5  $\mu$ g of pW597.3A plus 0.5  $\mu$ g of SV40 *neo*), and transfectants (MDA-453/M8, MDA-453/M12, and SK-BR-3/S3) were selected by continuous culture exposure to 800  $\mu$ g of G418 (Geneticin; GIBCO) per ml. Transfected BT-474 cultures were not cotransfected with SV40 *neo* but were instead subjected 24 h after transfection to selection cycles consisting of 10 days of continuous exposure to 2  $\mu$ g of muMab4D5 per ml, trypsinization, and passage in fresh (muMab4D5-free) medium for 24 h, followed by resumption of the muMab4D5 selection pressure. After the third selection cycle and relative to untransfected BT-474 cells, colonies resistant to the marked growth-inhibiting effect of muMab4D5 were observed. Two separate pools of resistant colonies (BT-474/pW-1 and BT-474/pW-2) were split from the primary culture after the third selection cycle and were subcultured according to the same muMab4D5 treatment regimen for two additional cycles before testing. Concentrated conditioned media and cell lysates from wild-type and transfected tumor cell lines (MKN7, MDA-MB-453, SK-BR-3, and BT-474) were prepared identically to the transfected COS-1 cells in order to examine protein expression. Aliquots of concentrated conditioned media and cell lysates were mixed in an equal volume of loading buffer (0.125 M Tris [pH 6.8], 20% glycerol, 2% SDS) either with or without 10 mM dithiothreitol (DTT) as indicated, electrophoresed on a 7% polyacrylamide-SDS denaturing gel, and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.). Size markers as indicated in figures were determined by comparing the running positions of prestained and unstained protein molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Md.). After prehybridization in Tris-buffered saline (TBS; 10 mM Tris [pH 8.0], 150 mM NaCl, 0.5% Tween 20) containing 1.5% nonfat dry milk powder, membranes were probed with either a guinea pig polyclonal antibody raised against the ECD of HER2 recombinantly produced in mammalian cells or another guinea pig polyclonal antibody raised against the C-terminal 17 amino acids of HER2 (9, 10). Polyclonal antibodies raised against the HER2 ECD were protein A purified from guinea pig serum and used at a concentration of 2  $\mu$ g/ml, while guinea pig antiserum against the HER2 C terminus was used at a 1:1,000 dilution. After several washes in TBS, membrane-bound antibodies were detected by using  $^{125}$ I-protein A (NEN Research Products, Boston, Mass.). Membranes were then washed again in TBS, dried, and filmed.

**Metabolic labeling, immunoprecipitation, and endoglycosidase digestion.** The differential processing and turnover of HER2 ECD was studied by using previously described techniques for the metabolic labeling and endoglycosidase (endo-H or endo-F) treatment of immunoprecipitated HER2 receptor (16). Cells were grown to 80% confluence in 35-mm-diameter culture dishes, then pulse-labeled in cysteine-free medium containing [ $^{35}$ S]cysteine (1 mCi/ml) for 30 min, and incubated for various times (chase) in 10% fetal calf serum-supplemented medium. After the chase period, cells were washed twice in PBS, lysed, and immunoprecipitated with an ECD-specific polyclonal antibody in combination with

protein A-agarose (Sigma). For endo-H (Boehringer Mannheim) digestion, the immunoprecipitated products were first resolubilized in lysis buffer containing 1% SDS and heated for 2 min. The supernatant was diluted 10-fold with lysis buffer containing endo-H (250 mU/ml) and incubated at 37°C for 4 h. Digestion products were reimmunoprecipitated and then electrophoresed (along with undigested products) on an SDS-7.5% polyacrylamide gel.

**Immunofluorescence of tumor cells.** Cultured HER2-over-expressing tumor cells were grown to subconfluence on coverslips, fixed with 3% formaldehyde, and permeabilized with 1% Triton X-100. Cells were then incubated with primary anti-HER2 antibodies. Either a guinea pig anti-HER2 ECD antiserum or muMab4D5 was used to label HER2 ECD. Either a guinea pig antiserum, a rabbit antiserum, or a mouse monoclonal antibody, each directed against the HER2 C terminus, was used to label full-length HER2. Following washes in PBS, the cells were incubated with an appropriate secondary goat antibody directed against either rabbit, mouse, or guinea pig immunoglobulin G. The secondary antibody was conjugated to either fluorescein or rhodamine. The cells were again washed, mounted, and then examined by using a Leitz Aristoplan fluorescence microscope.

## RESULTS

**Isolation and sequencing of HER2 ECD clones.** Three clones encoding the truncated HER2 ECD were isolated from cDNA libraries derived from the HER2-overexpressing cell lines BT-474 and SK-BR-3 (Fig. 1). The DNA sequences of these clones matched the known 5' untranslated and coding sequence of HER2 through base 1898 (from the initiating ATG codon in exon 1) and then diverged into a region of unique sequence terminating in a polyadenylation stretch (13 A's in clone  $\lambda$ 5A). The clones encode a reading frame that includes the first 633 amino acids of HER2 and diverges 20 amino acids 5' of the HER2 transmembrane domain. The divergent region begins with the sequence GTGAG, a consensus splice donor site, suggesting that the truncated transcript fails to splice at the 3' end of the exon and reads through for 165 bp to an alternative poly(A) site. The TGA in the putative splice site becomes the stop codon for the truncated transcript.

**Characterization of the divergent exon.** To determine whether the divergent sequence is contiguous in the genome with the immediately 5' common sequence, DNA from human placenta and BT-474 cells was subjected to PCR analysis using primers flanking the point of divergence. A major PCR band of 123 bp that hybridized to probe from the 3' region of the truncated HER2 cDNA clone was obtained from both placenta and BT-474 genomic DNA (Fig. 2). This amplified 123-bp band is the size expected if the two sequences are contiguous in the genome, substantiating the read-through of the HER2 splice site as the source of the divergent sequence. The significance of the minor hybridizing band occurring below the predominant 123-bp band is unknown, and PCR analysis using only one flanking primer yielded no 3' truncated HER2 hybridizing band (data not shown).

**Prevalence and cellular abundance of truncated HER2 transcript.** Previous Northern analyses had detected a 2.3-kb HER2 transcript in RNA isolated from the human gastric cancer cell line MKN7 that hybridized to 5' but not 3' HER2 cDNA probes (40). Blot hybridizations of RNA from BT-474 and MKN7 cells using probes specific for the 5' common and

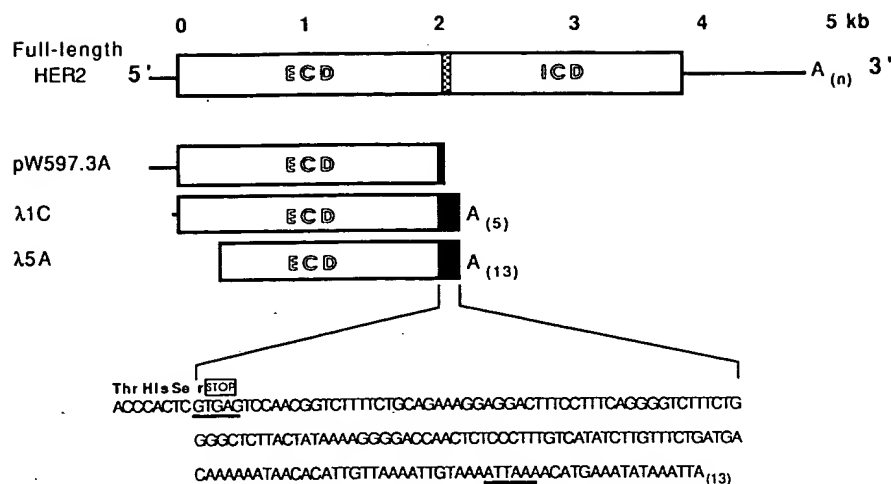


FIG. 1. Schematic diagram of truncated human HER2 cDNA clones containing 3' sequences divergent from full-length HER2 cDNA. Three clones were isolated from cDNA libraries from SK-BR-3 (pW597.3A) and BT-474 (λ1C and λ5A) human breast cancer cells. Horizontal lines represent untranslated regions from the 5' and 3' regions of the full-length HER2 cDNA. Open boxes represent either the ECD or the intracellular domain (ICD) of normal HER2; the stippled box represents the normal HER2 transmembrane domain. Solid boxes indicate the divergent region of the 3' end of the truncated HER2 cDNAs, the full sequence of which is shown in the expanded bracket highlighting the retained 5' splice donor site (GTGAG), the in-frame stop codon (TGA), and the poly(A) addition signal (ATTAAA).

3' divergent regions of the full-length and truncated HER2 transcripts show that a 4.6-kb band corresponding to the full-length transcript is found in both cell lines, while an obvious 2.3-kb band corresponding to the truncated transcript is found only in MKN7 RNA (Fig. 3). Fivefold overexposure reveals the presence of the 2.3-kb truncated HER2 transcript in BT-474 cells as well (data not shown). Previous detection of the 2.3-kb transcript in other cell lines, primarily those of human breast cancer origin, has been

equivocal because of the much greater abundance of the 4.6-kb HER2 band and the lack of a specific probe recognizing the 3' divergent sequence unique to the truncated transcript (1, 21, 41).

The relative amounts of the truncated and full-length HER2 transcripts were more readily quantitated by S1 nuclease assay. A 281-nt single-stranded fragment containing sequences spanning the point of divergence from the truncated HER2 clones was used as a probe. The truncated transcript was expected to protect 181 nt of this probe, while full-length transcript would protect 125 nt. Results of the S1 nuclease assay using RNA from cell lines MKN7, SK-OV-3, and BT-474 are shown in Fig. 4A. Quantitation of these data gave ratios of 1.1, 16, and 40 (full-length to truncated transcript levels) for the MKN7, SK-OV-3, and BT-474 RNA samples, respectively. Similar assays were performed to compare the relative abundance of the full-length and truncated HER2 transcripts present in human breast cancer

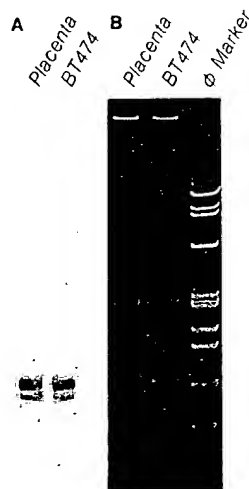


FIG. 2. Genomic contiguity of divergent cDNA sequence reflecting alternatively processed HER2 transcript. Human placenta and BT-474 genomic DNA was PCR amplified by using primers flanking the point of divergence (45 bp of common exonic sequence and 78 bp of putative intronic sequence). Aliquots of the reaction mixture were electrophoresed in a 12% polyacrylamide gel with φX174 replicative-form DNA/HaeIII (Bethesda Research Laboratories) restriction fragments as markers, stained with ethidium bromide, and photographed (B). DNA was transferred to a nylon membrane and probed with a fragment from the 3' region of the truncated HER2 cDNA clone (A).

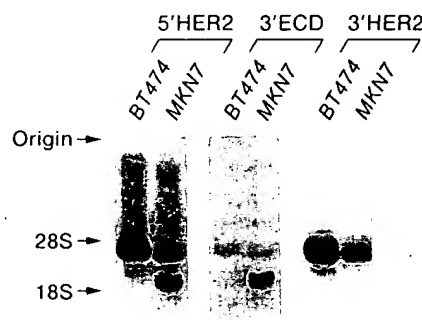


FIG. 3. Specificity of the 3' region of truncated HER2 cDNA for the 2.3-kb RNA transcript. Identical Northern membranes containing BT-474 and MKN7 total RNA at 10 μg per lane were probed with a 5' HER2 cDNA probe (5' HER2), a 3' HER2 cDNA probe (3' HER2), and a probe primarily specific for the 3' region of the truncated HER2 cDNA (3' ECD). Positions of 18S and 28S rRNAs, determined from the ethidium bromide-stained RNA gels, are indicated.

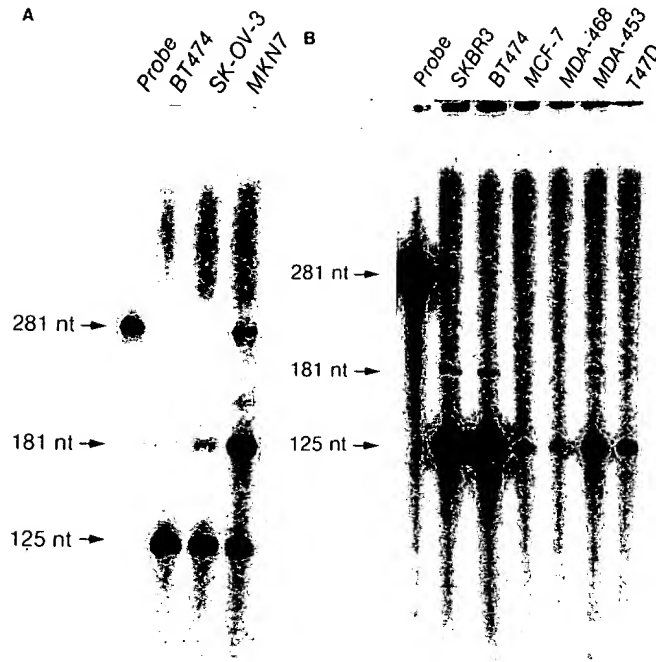


FIG. 4. S1 nuclease protection assay quantitating relative tumor cell abundance of 2.3-kb HER2 ECD mRNA. A single-stranded DNA fragment containing sequences spanning the point of divergence in the truncated HER2 cDNA clones was primed by an M13 reverse primer to generate the radiolabeled 281-nt probe used in the S1 protection assay conditions as described in Materials and Methods. Thirty micrograms of total RNA from the indicated cell lines was subjected to S1 assay, and the protected products were separated by electrophoresis on 5% polyacrylamide-8 M urea gels. Arrows indicate gel positions of the intact 281-nt probe, the 181-nt fragment protected by the sequence divergence within the truncated HER2 transcript, and a 125-nt exonic portion of that probe fragment protected only by full-length HER2 transcript. The more prolonged autoradiograph exposure shown in panel B was necessary to detect the HER2 ECD-derived band (181 nt) from MCF-7 and T47D cells; an even longer autoradiograph exposure was necessary to detect this truncated transcript from MDA-468 cells (not shown).

cell lines known to greatly overexpress (BT-474 and SK-BR-3), moderately overexpress (MDA-MB-453 and T47D), or normally express (MCF-7 and MDA-MB-468) HER2 receptor (Fig. 4B). Irrespective of HER2 receptor level varying 30-fold among these breast cancer cell lines, the ratio of full-length to truncated HER2 mRNA levels was essentially constant and equal to 40.

**Cellular expression of truncated HER2 protein.** Immunoblotting of COS-1 cells transfected with the HER2 ECD expression clone (pW597.3A) was performed to examine the size and pattern of expression of the truncated HER2 protein. Samples from the concentrated serum-free conditioned medium and COS-1 cell extract were analyzed by SDS-polyacrylamide gel electrophoresis and stained with anti-HER2 ECD antibodies (Fig. 5A). Under reducing conditions, conditioned medium showed a 110-kDa band, while the band from the cell extract migrated at approximately 100 kDa. Since the molecular size of the protein based on translation of the HER2 ECD cDNA sequence is about 70 kDa, both the secreted and cytosolic forms appeared to be glycosylated to various extents (6, 40). In agreement with the known disulfide-linked cysteines in the HER2 ECD, the unreduced and more compact form of the truncated protein

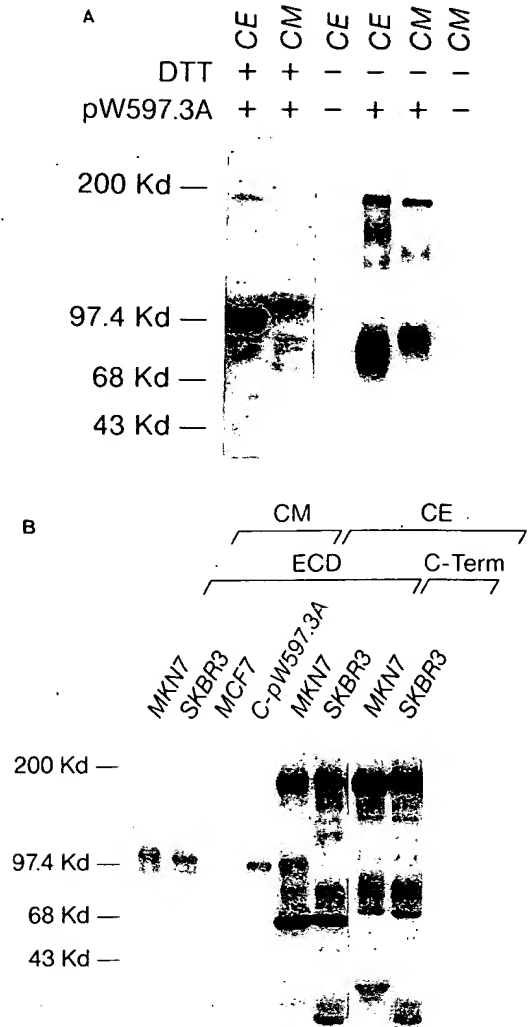


FIG. 5. Size and pattern of expression of truncated HER2 protein. (A) Reduced (DTT +) and nonreduced (DTT -) aliquots of conditioned media (CM) or cell extract (CE) from COS-1 cells transiently transfected with the HER2 ECD expression vector pW597.3A were electrophoresed in an SDS-7% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, probed with polyclonal antibodies against the HER2 ECD, and detected by  $^{125}$ I-protein A. (B) DTT-reduced aliquots of conditioned media (CM) or cell extracts (CE) from the indicated cell lines (including the transfected COS-1 cells, C-pW597.3A) were electrophoresed, transferred to a membrane, probed with either polyclonal antibody against the HER2 ECD (ECD) or polyclonal antibody against the HER2 C terminus (C-Term), and detected by  $^{125}$ I-protein A. The immunoreactive bands at 60 to 70 kDa in MKN7 and SK-BR-3 cell extracts, as well as the other lower-molecular-weight bands, represent nonspecific (non-HER2 protein) detection by polyclonal antibody, since these bands are present with near-equal intensity in MCF-7 cell extract (not shown). Protein molecular weight standards were used to determine the positions of size markers shown on the left.

migrated more rapidly than did the reduced HER2 ECD (Fig. 5A).

Immunoblotting of cell lines (MKN7 and SK-BR-3) with greatly overexpressed HER2 receptor and different ratios of full-length to truncated HER2 transcript was performed to compare the size and pattern of the native HER2 protein expressed in these tumor cell lines. Extracts from both cell

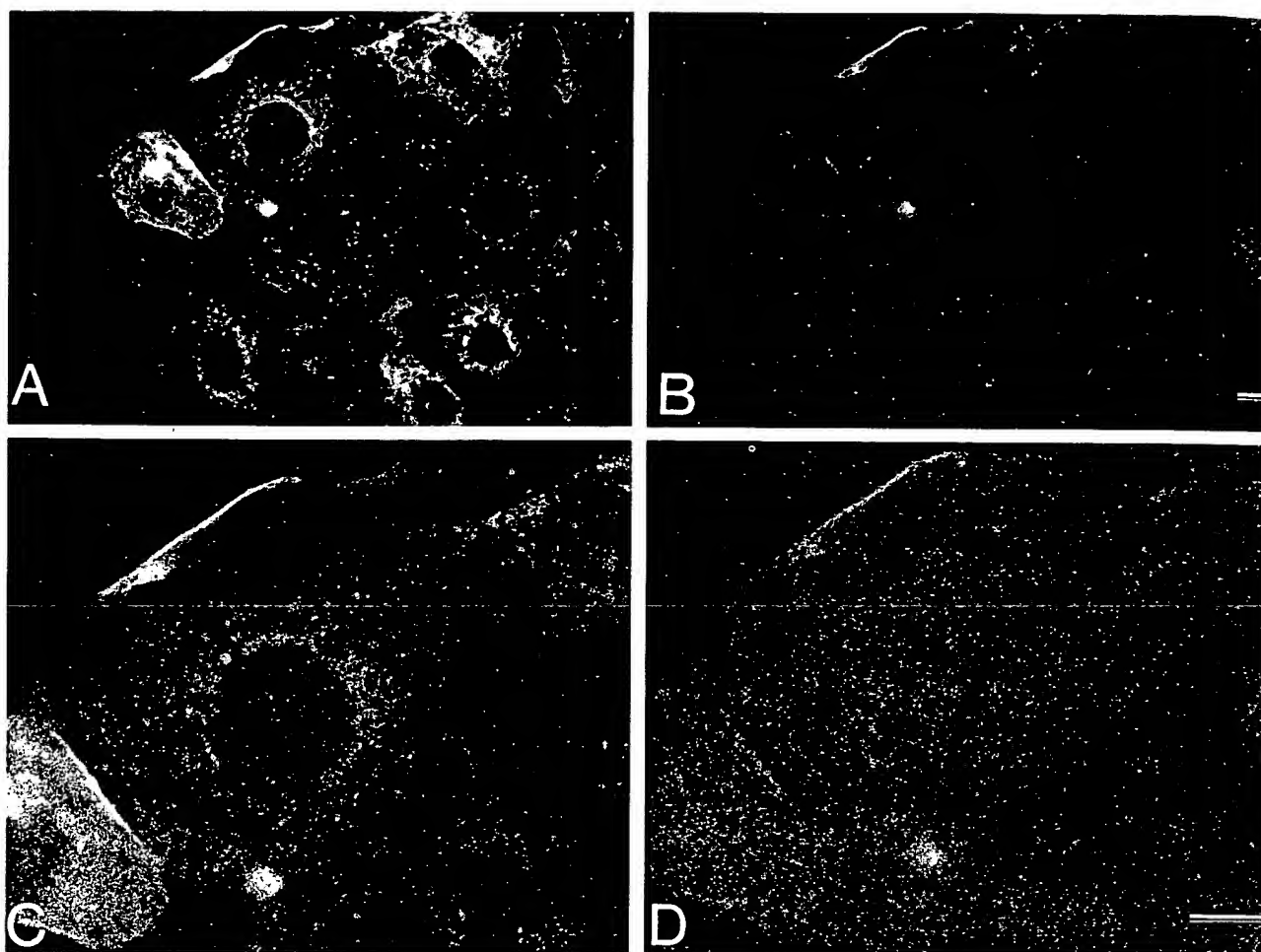


FIG. 6. Double immunofluorescence labeling of MKN7 cells overexpressing full-length HER2 (185 kDa) and HER2 ECD (100 kDa) demonstrating differential cellular localization of the truncated and full-length receptor proteins. As described in Materials and Methods, cultured MKN7 cells were labeled with both an antibody directed against the HER2 ECD domain and an antibody directed against the cytoplasmic C-terminal domain of HER2. Diffuse cell surface membrane fluorescence from rhodamine-labeled full-length HER2 receptor is seen in panels B (low power) and D (high power). In contrast, panels A (low power) and C (high power) show the same cells with intense perinuclear as well as diffuse surface membrane fluorescence from fluorescein-labeled HER2 ECD. The bars represent 10  $\mu$ m.

lines contained equal and abundant levels of intact (185-kDa) HER2 receptor when probed with anti-HER2 ECD antibody (Fig. 5B). As well, the concentrated conditioned medium from SK-BR-3 cells (having low 2.3-kb transcript levels relative to 4.6-kb HER2 mRNA) contained as much 110-kDa immunoreactive protein as did that from MKN7 cells (with high levels of 2.3-kb HER2 mRNA), implicating proteolysis of the abundant cell surface receptor as one mechanism for soluble HER2 ECD production (see Discussion). In contrast, only the MKN7 cell extract contained abundant 100-kDa HER2 ECD protein like that found in transfected COS-1 cells (Fig. 5B). As expected, when probed with antibody specific for the C-terminal region of the intact HER2 protein, the 100-kDa HER2 ECD band from the MKN7 cell extract was no longer detectable (Fig. 5B). Thus, the abundance of 100-kDa HER2 ECD protein in the MKN7 cell extract corresponds to the amount of truncated 2.3-kb HER2 mRNA expressed in these cells.

Two different procedures were performed to demonstrate the intracellular localization of 100-kDa HER2 ECD in MKN7 cells: (i) cultured cells were subjected to trypsiniza-

tion prior to HER2 immunoblot assay of cell extracts, and (ii) cells were double immune labeled with C-terminus- and ECD-specific anti-HER2 antibodies. Trypsin treatment of MKN7 cultures (0.5 and 2.5 mg of trypsin per ml in 1 mM EDTA at 37°C for 15 to 30 min) produced partial cleavage followed by complete loss of intact (185-kDa) HER2 receptor with no significant decline in extract levels of truncated (100-kDa) HER2 protein (data not shown), indicating that the intracellular location of MKN7 HER2 ECD protected it from exposure to a proteolytic attack capable of completely degrading cell surface HER2 receptor. To localize intracellular HER2 ECD relative to full-length HER2 in MKN7 cells, double immunofluorescence studies were performed with antibodies recognizing five different epitopes within either the extracellular or cytoplasmic domain of HER2: two different antibodies specific for separate HER2 C-terminus epitopes in the cytoplasmic domain and three different anti-ECD antibodies specific for distinct N-terminus epitopes. As shown (Fig. 6A and C), MKN7 cells labeled with either a polyclonal antiserum or a monoclonal antibody (muMAb4D5) directed against the HER2 ECD showed dif-



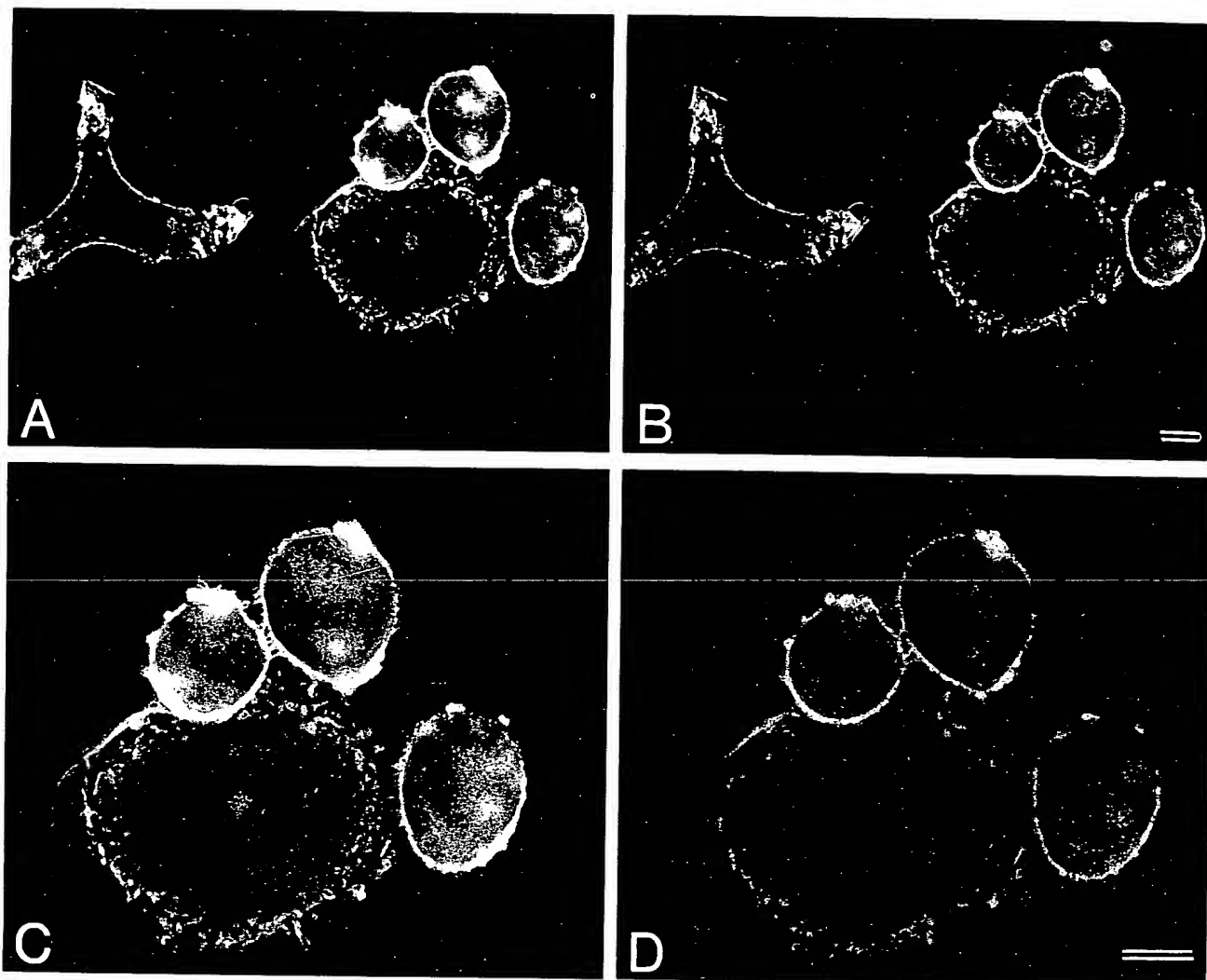


FIG. 7. Double immunofluorescence labeling of SK-BR-3 cells overexpressing full-length HER2 but not HER2 ECD. Cultured SK-BR-3 cells were labeled with the same ECD-specific antibody (A and C) and antibody directed against the HER2 cytoplasmic domain (B and D) as described for Fig. 6; fluorescence was visualized under low-power (A and B) and high-power (C and D) magnification. With these control cells, both antibodies demonstrate membrane labeling without significant intracellular fluorescence. The bars represent 10  $\mu$ m.

fuse surface membrane-localized immunofluorescence in addition to a more intense pattern of intracellular immunofluorescence localized to the perinuclear region. When these same cells were simultaneously labeled with either antipeptide antiserum or a monoclonal antibody directed against the HER2 C terminus, diffuse surface membrane immunofluorescence predominated (Fig. 6B and D). Double immunolabeling of control SK-BR-3 cells, known to overexpress full-length HER2 but not HER2 ECD, showed pronounced membrane labeling without significant intracellular fluorescence by either the C-terminus- or N-terminus-specific antibodies (Fig. 7). Of note, when transfected and HER2 ECD-overexpressing SK-BR-3 cells (see Materials and Methods) were similarly labeled, intracellular fluorescence was observed with the N-terminus-specific antibodies but not with the C-terminus-specific antibodies (results not shown). The trypsinization studies together with the double immunofluorescence labeling studies indicate that a major portion of the 2.3-kb transcript-encoded HER2 ECD localizes intracellularly.

Metabolic labeling and endoglycosidase (endo-H or endo-F) treatment of HER2 immunoprecipitates from MKN7 cells was performed to detect differential processing and turnover by intracellularly sequestered HER2 ECD in comparison with surface membrane-bound full-length receptor. The pulse-chase result shown in Fig. 8A demonstrates that HER2 ECD does not mature beyond the 100-kDa form detected immediately upon  $^{35}$ S labeling, unlike full-length HER2, which characteristically shows more extensive glycosylation and processing of its 170-kDa precursor form into the mature 185-kDa glycoprotein (16). Figure 8B demonstrates that the 100-kDa HER2 ECD is sensitive to endo-H digestion following pulse-chase (consistent with high-mannose-type N-linked glycosylation), resulting in the 75-kDa HER2 ECD digestion products. In contrast, while the 170-kDa HER2 precursor is endo-H sensitive, the mature 185-kDa HER2 receptor is endo-H resistant, a result of Golgi processing of the high-mannose-type full-length precursor into the complex-type 185-kDa mature glycoprotein (16). Similar results showing differential processing of 185-kDa



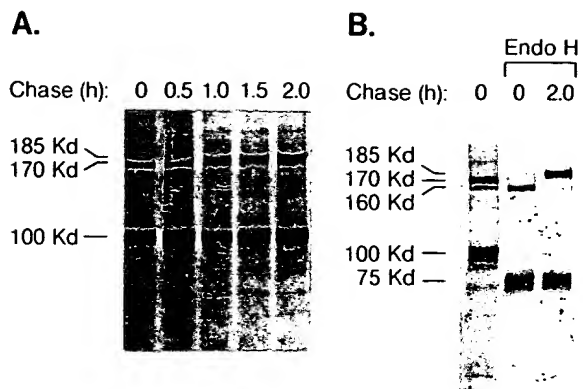


FIG. 8. Metabolic labeling of MKN7 cells and endo-H digestion of HER2 immunoprecipitates. MKN7 cultures were pulsed-labeled with [ $^{35}$ S]cysteine for 30 min, chased for various intervals up to 2 h prior to cell lysis, and then immunoprecipitated with a HER2 ECD-specific polyclonal antibody. Labeled immunoprecipitates were analyzed before (A) and after (B) endo-H digestion by SDS-7.5% polyacrylamide gel electrophoresis and autoradiography. Protein molecular weight standards were used to determine the positions of size markers shown on the left.

HER2 and HER2 ECD were obtained with endo-F treatment of MKN7 HER2 immunoprecipitates.

**Induction of muMab4D5 resistance by intracellular overexpression of HER2 ECD.** The observation of increased levels of 2.3-kb HER2 transcript in tumor cell lines showing atypical resistance to the growth-inhibiting effects of anti-HER2 antibodies suggested that intracellular overexpression of HER2 ECD might directly alter the growth response of cells exposed to muMab4D5 (see Discussion). In the first test of this hypothesis, MDA-MB-453 cells cotransfected with HER2 ECD and *neo* expression vectors were clonally selected and amplified on the basis of G418 resistance, examined for cell extract overexpression of 100-kDa HER2 ECD protein, and then tested for sensitivity to a growth-inhibiting dose of the anti-HER2 monoclonal antibody muMab4D5 (10, 17, 35). In two fully characterized MDA-MB-453 clones (MDA-453/M8 and MDA-453/M12), cell extract immunoblots confirmed the presence of HER2 ECD protein, which was only faintly detectable in untransfected MDA-MB-453 cells (Fig. 9). Both of these transfected clones showed reduced sensitivity to a known growth-inhibiting concentration of muMab4D5 (Fig. 10). In a second test of this hypothesis, BT-474 cells were transfected with the HER2 ECD expression vector but without cotransfection of the G418-selectable resistance marker. Transfectants were then selected by exposure to a growth-inhibiting concentration (2  $\mu$ g/ml) of muMab4D5 as described in Materials and Methods. Control BT-474 cells, which contain minimal intracellular HER2 ECD but produce in their conditioned medium amounts of soluble ECD similar to those produced by MKN7 cells (data not shown), were also subjected to selection pressure by 2  $\mu$ g of muMab4D5 per ml. After more than 15 weeks of antibody exposure, control BT-474 cells continued to show the same marked degree of cytostasis by muMab4D5 as previously reported (36), with no evidence for the emergence of muMab4D5 resistance. In contrast, pW597.3A-transfected and muMab4D5-selected BT-474 cells not only overexpressed 100-kDa HER2 ECD intracellularly (Fig. 9) but also proliferated more than twice as fast as control BT-474 cells (0.74 doubling per day versus 0.35

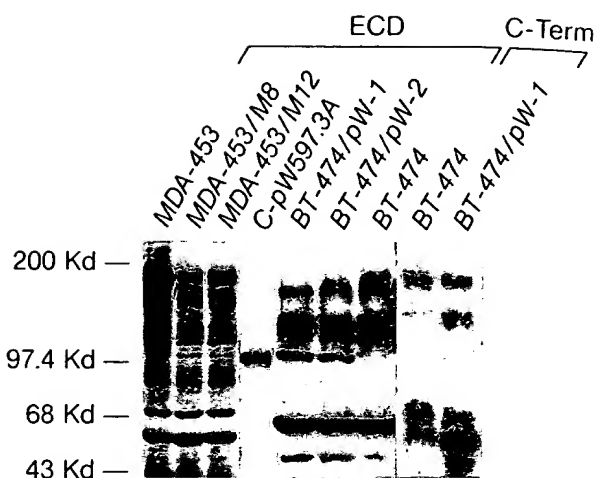


FIG. 9. Overexpression of HER2 ECD in BT-474 and MDA-453 tumor cells. MDA-453 and BT-474 cells were transfected with the HER2 ECD expression vector (pW597.3A) to yield separate cultures of permanent transfectants (MDA-453/M8, MDA-453/M12, BT-474/pW-1, and BT-474/pW-2) as described in Materials and Methods. DTT-reduced aliquots of extracts from untransfected and transfected cells, including transfected COS cells (C-pW597.3A), were electrophoresed, transferred to a membrane, and probed with either HER2 ECD- or HER2 C-terminus (C-Term)-specific polyclonal antibodies, and protein bands were identified by  $^{125}$ I-protein A as described for Fig. 5. As shown, the differentially stained 100-kDa HER2 ECD band (located by the single intense band from the C-pW597.3A extract) is overexpressed only in the transfected cells, while full-length HER2 receptor (the intense band just below the 200-kDa marker) is overexpressed in both control and transfected tumor cells.

doubling per day) and demonstrated no significant growth inhibition on continuous culture exposure to 2  $\mu$ g of muMab4D5 per ml (Fig. 10).

## DISCUSSION

Results presented here demonstrate that a truncated 2.3-kb transcript from the HER2 proto-oncogene is produced by alternative processing of the primary HER2 receptor transcript. The 5' 2.1-kb segment of the truncated HER2 transcript is identical to the 5' end of the full-length 4.6-kb HER2 transcript; however, the 3' end diverges in the truncated transcript, revealing an exonic extension with an in-frame stop codon and a poly(A) addition site. Thus, unlike the alternate exonic splicing mechanism most commonly described for other transmembrane receptor systems, including the rat EGF receptor (33), production of the truncated HER2 transcript uses an alternative polyadenylation signal that permits reading into the HER2 intron, like that recently reported for the chicken EGF receptor system (11). The truncated HER2 transcript encodes a 100-kDa form of the 185-kDa transmembrane receptor that, with the exception of 20 C-terminal amino acids, contains the entire HER2 ECD. This HER2 ECD protein is fully recognized by N-terminus-specific anti-HER2 antibodies, and the alternatively processed ECD-encoding transcript is present at variable levels in all eight HER2-expressing cancer cell lines examined.

Observation of a 2.3-kb HER2 transcript in the HER2-overexpressing gastric cancer cell line MKN7 that hybridized with 5' but not 3' HER2 cDNA probes was initially

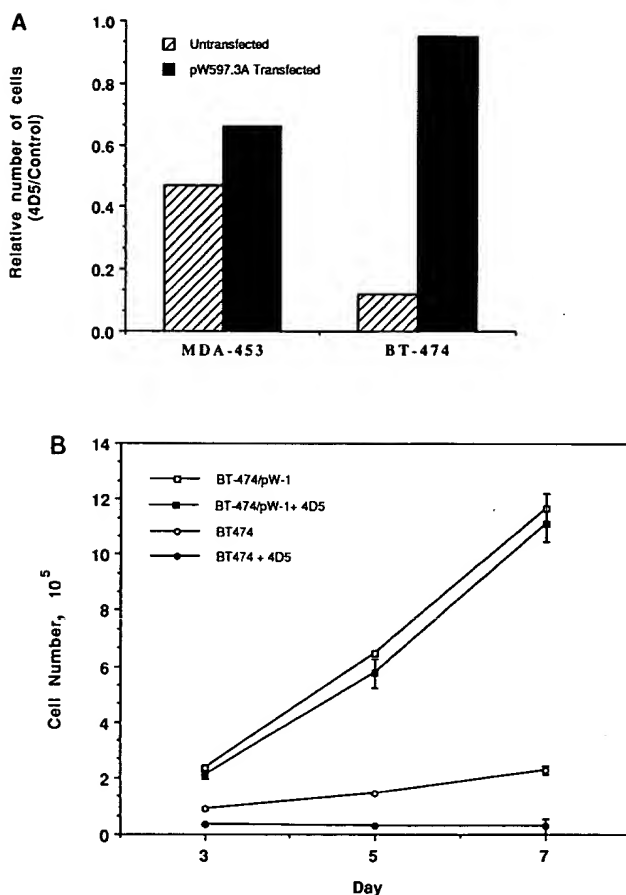


FIG. 10. Resistance of HER2-overexpressing tumor cells to muMab4D5 induced by the transfected overexpression of HER2 ECD. Control and pW597.3A-transfected MDA-453 (MDA-453/M12) and BT-474 (BT-474/pW-1) cells ( $10^3$ ) were plated into triplicate 25-cm<sup>2</sup> culture flasks on day 0, with or without the addition of 2  $\mu$ g of muMab4D5 per ml. (A) Flasks were harvested, and mean total cell counts were determined on day 7, with results averaged from duplicate experiments and shown as the ratio of muMab4D5-treated to untreated cell counts for both transfected and untransfected tumor cells. (B) To compare the different muMab4D5 treatment responses over time, mean cell counts ( $\pm$  standard error of the mean) for transfected and untransfected BT-474 cells were determined after 3, 5, and 7 days of growth in culture.

reported by Yamamoto et al. (40). Evidence from other groups for the existence of a truncated HER2 transcript by Northern blotting of RNA from HER2-overexpressing human breast cancer cell lines has been inconclusive, in part because of the predominance of the normal 4.6-kb HER2 transcript (1, 21, 41). The relative abundance of the full-length versus the truncated HER2 transcript varies greatly in the different breast, ovarian, and gastric cancer cell lines. In MKN7 cells, the ratio of full-length to truncated transcript is 1.1. By comparison, all six of the breast cancer cell lines (irrespective of their 30-fold variation in overall HER2 expression) exhibit a ratio of 40, while the SK-OV-3 ovarian cancer cells have a ratio of 16. At present, the cellular factors governing the relative amounts of the two HER2 transcripts are unknown.

Clinical analysis has detected HER2 ECD protein in the blood of patients with HER2-overexpressing tumors (23), and several reports have characterized the soluble HER2

ECD protein found in media conditioned by HER2-overexpressing SK-BR-3 and BT-474 cells (1, 24, 41). However, the mechanism accounting for this soluble HER2 ECD protein has not been fully established. Pulse-chase experiments suggest that the HER2 membrane receptor on SK-BR-3 cells is proteolytically cleaved to release soluble ECD (41). Furthermore, the fact that soluble HER2 ECD can be shed by proteolytic cleavage of surface receptor may be deduced from studies using HER2-negative NIH 3T3 cells transfected with a full-length HER2 cDNA expression vector (22). Although full-length HER2 cDNA should not generate a truncated HER2 transcript of the type that we have identified, extracellular HER2 ECD is abundantly produced by HER2-transfected NIH 3T3 cells. Thus, it is possible that the major proportion of extracellular HER2 ECD protein is produced by proteolytic cleavage of overexpressed 185-kDa surface receptor.

In contrast to extracellular HER2 ECD generated by proteolytic shedding of surface receptor, intracellular 100-kDa HER2 ECD produced by truncated (2.3-kb) HER2 transcript appears to undergo differential trafficking between endoplasmic reticulum and Golgi, leading to its perinuclear accumulation. This conclusion is supported by four different observations: (i) Western immunoblots show that while MKN7, BT-474, and SK-BR-3 cells all produce comparable amounts of soluble HER2 ECD, MKN7 cells produce increased levels of cell-associated 100-kDa HER2 ECD, consistent with their near 40-fold excess level of truncated HER2 transcript relative to BT-474 and SK-BR-3 cells; (ii) MKN7 cells trypsinized until their surface 185-kDa HER2 receptor is no longer detectable by Western blotting still contain excess levels of 100-kDa HER2 ECD in their cell extracts; (iii) double immunofluorescence labeling of MKN7 cells using C-terminus- and ECD-specific anti-HER2 antibodies shows that the overexpressed HER2 ECD is intracellular and concentrated within the perinuclear region; and (iv) metabolic labeling and endoglycosidase digestion indicate that HER2 ECD is composed of high-mannose-type *N*-glycan, in contrast to the complex-type *N*-glycan present in mature 185-kDa HER2. This endo-H sensitivity and arrested maturation and processing of HER2 ECD *N*-glycan may signify that newly synthesized 100-kDa HER2 ECD undergoes differential trafficking between the endoplasmic reticulum and Golgi compartments compared with full-length receptor. Of interest, the apparent perinuclear site of sequestration for HER2 ECD is similar to the intracellular site of routing for 185-kDa surface receptor following binding by either ligand or any one of several growth-inhibiting (but not growth-stimulating) anti-HER2 antibodies (2, 3); this also appears to be the intracellular routing for receptor-bound muMab4D5 (data not shown). Together, these findings not only indicate that 100-kDa HER2 ECD may be differentially routed between the endoplasmic reticulum and Golgi compartments compared with newly synthesized full-length HER2 but also point to a possible subcellular site of interaction between newly synthesized HER2 ECD and membrane-activated and internalized 185-kDa HER2 receptor.

The possible biological significance of this overexpressed intracellular HER2 ECD was first suggested by the atypical resistance profiles of cultured MKN7 and SK-OV-3 cells exposed to growth-inhibiting concentrations of muMab4D5. Except for these cell lines, the ability of anti-HER2 monoclonal antibodies such as muMab4D5 to inhibit growth of HER2-overexpressing tumor cells appears to correlate well with 185-kDa HER2 receptor levels (10, 17, 35). For example, tumor cells BT-474 and SK-BR-3, with approximately

25-fold-greater HER2 surface receptor level relative to MCF-7, experience 67% growth inhibition in the presence of anti-HER2 monoclonal antibodies (mean responses to three different growth-inhibiting monoclonal antibodies [4D5, 3H4, and 7F3] relative to control cell responses). This sensitivity to different anti-HER2 monoclonal antibodies occurs despite excessive extracellular shedding of soluble HER2 ECD by these cells. Growth of MCF-7 and other low-HER2-expressing cell lines (e.g., MDA-MB-468) is essentially unaffected by these anti-HER2 monoclonal antibodies. In contrast, SK-OV-3 and MKN7 cells, with their 15-fold overexpression of HER2 surface receptor (but 2.5- to 40-fold relative excess of truncated HER2 transcript levels compared with BT-474 and SK-BR-3 cells), respond like low-expressing cells and show only 16 and 0% growth inhibition, respectively, on exposure to these same anti-HER2 antibodies. The overexpressed 185-kDa receptor on anti-HER2-resistant MKN7 cells is bound and internalized by these monoclonal antibodies much like that of SK-BR-3 and BT-474 cells (35; data not shown), suggesting that their resistance is not accounted for by lack of receptor availability or ability to internalize. To determine whether there is a mechanistic association between anti-HER2 resistance and overexpressed intracellular HER2 ECD, transfection studies were performed.

HER2-overexpressing breast cancer cell lines sensitive to muMab4D5 were transfected with the HER2 ECD. The two HER2 ECD-transfected and -overexpressing MDA-MB-453 clones assayed after G418 selection were found to have acquired significant resistance to muMab4D5. To test more rigorously for a dependent relationship between HER2 ECD overexpression and muMab4D5 resistance, BT-474 cells (the most sensitive of our HER2-overexpressing cells) were selected after transfection by exposure to a completely cytostatic dose of muMab4D5 (2 µg/ml). The resulting BT-474 transfectants not only overexpressed intracellular 100-kDa HER2 ECD but also demonstrated more rapid growth in culture and complete resistance to growth inhibition by muMab4D5. These transfection results suggest that the intracellular overexpression of truncated HER2 transcript and 100-kDa HER2 ECD can inhibit the normal cytostatic response of HER2-overexpressing cells to muMab4D5 and provide a likely explanation for the observed natural resistance of SK-OV-3 and MKN7 cells to anti-HER2 monoclonal antibodies.

In our studies, it is not likely that either the acquired or native resistance of HER2-overexpressing cancer cells to muMab4D5 is produced by the presence of or interaction with heregulin, a specific high-affinity ligand for the 185-kDa HER2 receptor that has been recently described (15, 32), although it would be of interest to learn whether any interaction can occur between heregulin and the 100-kDa HER2 ECD. Since heregulin transcripts are not detectable in the eight different breast, ovarian, and gastric cancer cell lines used for this study (data not shown), the presence of heregulin cannot explain any of our reported findings. The mechanism by which HER2 ECD overexpression results in resistance to muMab4D5 remains unclear. One hypothesis is that the perinuclear accumulation of HER2 ECD, as demonstrated in MKN7 cells, somehow interferes with internalized complexes consisting of muMab4D5 and 185-kDa HER2. Other data from related receptor systems also support a biological role for HER2 ECD.

The finding of soluble ECD forms in many transmembrane receptor systems generated by proteolytic cleavage and/or alternative transcriptional processing has prompted the sug-

gestion that these proteins share a common physiological function. Avian, rodent, and human ECD forms of the EGF receptor have been identified and found overexpressed in embryonic and adult tissues (5, 11, 33). At present, however, only the avian form is known to be generated by the same unusual alternate polyadenylation mechanism that produces the truncated HER2 transcript that we find in human tumor cells (11), and there have been no prior reports on the existence and significance of intracellularly localized HER2 ECD in any mammalian species. Studies in progress are looking for evidence of a physical interaction between 100-kDa HER2 ECD and 185-kDa HER2 receptor within HER2-overexpressing cells, since truncated and normal EGF receptors are known to heterodimerize and dominantly suppress the activation and signaling of normal EGF receptor homodimers (5, 11, 19). The intracellular formation of such heterodimers in HER2-overexpressing tumor cells that overproduce a 2.3-kb transcript, like MKN7 cells, might explain the ability of muMab4D5 to bind and internalize the 185-kDa HER2 receptor but not transduce its growth inhibitory effect. Our results underscore the urgency for a better understanding of the physiological role of the HER2 receptor system and provide evidence that intracellular sequestration of HER2 ECD may alter the growth characteristics of HER2-expressing human tumors.

#### ACKNOWLEDGMENTS

This work was supported by grants CA-44768 and CA-36773 from the National Cancer Institute and in part by the Hazel P. Monroe Memorial Fund and the UCSF Breast Cancer Research Fund.

We thank Haleh Asgari, Janeen Wyatt, and Xiao Hui Xiong for excellent technical assistance.

#### REFERENCES

- Alper, O., K. Yamaguchi, J. Hitomi, S. Honda, T. Matsushima, and K. Abe. 1990. The presence of *c-erbB-2* gene product-related protein in culture medium conditioned by breast cancer cell line SK-Br-3. *Cell Growth Differ.* 1:591-599.
- Bacus, S. S., E. Huberman, D. Chin, K. Kiguchi, S. Simpson, M. Lippman, and R. Lupu. 1992. A ligand for the *erbB-2* oncogenic product (gp30) induces differentiation of human breast cancer cells. *Cell Growth Differ.* 3:401-411.
- Bacus, S. S., I. Stancovski, E. Huberman, D. Chin, E. Hurwitz, G. B. Mills, A. Ullrich, M. Sela, and Y. Yarden. 1992. Tumor inhibitory monoclonal antibodies to the HER-2/Neu receptor induce differentiation of human breast cancer cells. *Cancer Res.* 52:2580-2589.
- Bargmann, C. I., M.-C. Hung, and R. Weinberg. 1986. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. *Nature (London)* 319:226-230.
- Basu, A., R. M. Raghunath, S. Bishayee, and M. Das. 1989. Inhibition of tyrosine kinase activity of the epidermal growth factor (EGF) receptor by a truncated receptor form that binds to EGF: role for interreceptor interaction in kinase regulation. *Mol. Cell. Biol.* 9:671-677.
- Coussens, L., T. L. Yang-Feng, Y.-C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* 230:1132-1139.
- Downing, J. R., M. F. Roussel, and C. J. Sherr. 1989. Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. *Mol. Cell. Biol.* 9:2890-2896.
- Engelhorn, S., and R. W. Raab. 1991. Rapid electroblotting of small fragments from polyacrylamide gels. *BioTechniques* 11:594-596.
- Fendly, B. M., C. Kotts, D. Vetterlein, G. D. Lewis, M. Winget,

- M. E. Carver, S. R. Watson, J. Sarup, S. Saks, A. Ullrich, and H. M. Shepard. 1990. The extracellular domain of HER2/*neu* is a potential immunogen for active specific immunotherapy of breast cancer. *J. Biol. Response Modif.* 9:449-455.
10. Fendly, B. M., M. Winget, R. M. Hudziak, M. T. Lipari, M. A. Napier, and A. Ullrich. 1991. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/*neu* gene product. *Cancer Res.* 50:1550-1558.
11. Flickinger, T. W., N. J. Maihle, and H.-J. Kung. 1992. An alternatively processed mRNA from the avian *c-erbB* gene encodes a soluble, truncated form of the receptor that can block ligand-dependent transformation. *Mol. Cell. Biol.* 12:883-893.
12. Gubler, U., and B. J. Hoffmann. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.
13. Gullick, W. J., L. B. Love, C. Wright, D. M. Barnes, B. Gusterson, A. L. Harris, and D. G. Altman. 1991. *c-erbB-2* protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br. J. Cancer* 63:434-438.
14. Hancock, M. C., B. C. Langton, T. Chan, P. Toy, J. J. Monahan, R. P. Mischak, and L. K. Shawyer. 1991. A monoclonal antibody against the *c-erbB-2* protein enhances the cytotoxicity of cis-diaminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res.* 51:4575-4580.
15. Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W.-J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vanden. 1992. Identification of heregulin, a specific activator of p185<sup>erbB2</sup>. *Science* 256:1205-1210.
16. Huang, S. S., H. A. Koh, Y. Konish, L. D. Bullock, and J. S. Huang. 1990. Differential processing and turnover of the oncogenically activated *neulerbB2* gene product and its normal cellular counterpart. *J. Biol. Chem.* 265:3340-3346.
17. Hudziak, R. M., G. D. Lewis, M. Winget, B. M. Fendly, H. M. Shepard, and A. Ullrich. 1989. p185<sup>HER2</sup> monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol. Cell. Biol.* 9:1165-1172.
18. Ishii, S., F. Imamoto, Y. Yamanashi, K. Toyoshima, and T. Yamamoto. 1987. Characterization of the promoter region of the human *c-erbB-2* protooncogene. *Proc. Natl. Acad. Sci. USA* 84:4374-4378.
19. Kashles, O., Y. Yarden, R. Fischer, A. Ullrich, and J. Schlessinger. 1991. A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. *Mol. Cell. Biol.* 11:1454-1463.
20. King, C. R., M. H. Kraus, and S. A. Aaronson. 1985. Amplification of a novel *v-erbB*-related gene in a human mammary carcinoma. *Science* 229:974-976.
21. Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King. 1987. Overexpression of the EGF receptor-related proto-oncogene *erbB-2* in human mammary tumor cell lines by different molecular mechanisms. *EMBO J.* 6:605-610.
22. Langton, B. C., M. C. Crenshaw, L. A. Chao, S. G. Stuart, R. W. Akita, and J. E. Jackson. 1991. An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the *c-erbB-2* (HER-2/*neu*) oncogene. *Cancer Res.* 51:2593-2598.
23. Leitzel, K., Y. Teramoto, E. L. Sampson, G. A. Wallingford, S. Weaver, L. Dcmcro, H. Harvey, and A. Lipton. 1991. Elevated *c-erbB-2* levels in the serum and tumor extracts of breast cancer patients. *Proc. Am. Assoc. Cancer Res.* 32:997.
24. Lin, Y. J., and G. M. Clinton. 1991. A soluble protein related to the HER-2 proto-oncogene product is released from human breast carcinoma cells. *Oncogene* 6:639-643.
25. Liu, E., G. Santos, W. Lee, C. K. Osborne, and C. Benz. 1989. Effects of *c-myc* overexpression on the growth characteristics of MCF-7 human breast cancer cells. *Oncogene* 4:979-984.
26. Loughnan, M. S., C. J. Sanderson, and G. J. V. Nossal. 1988. Soluble interleukin 2 receptors are released from the cell surface of normal murine B lymphocytes stimulated with interleukin 5. *Proc. Natl. Acad. Sci. USA* 85:3115-3119.
27. Lupu, R., R. Colomer, G. Zugmaier, J. Sarup, M. Shepard, D. Slamon, and M. E. Lippman. 1990. Direct interaction of a ligand for the *erbB-2* oncogene product with the EGF receptor and p185<sup>erbB-2</sup>. *Science* 249:1552-1555.
28. Maihle, N. J., T. W. Flickinger, M. A. Raines, M. L. Sanders, and H.-J. Kung. 1991. Native avian *c-erbB* gene expresses a secreted protein product corresponding to the ligand-binding domain of the receptor. *Proc. Natl. Acad. Sci. USA* 88:1825-1829.
29. McKenzie, S. E., M. A. Keller, D. L. Cassel, A. D. Schreiber, E. Schwartz, S. Surrey, and E. F. Rappaport. 1992. Characterization of the 5'-flanking transcriptional regulatory region of the human Fcγ receptor gene, FcγRIIA. *Mol. Immunol.* 29:1165-1174.
30. Mosley, B., P. Beckmann, C. J. March, R. L. Idzerda, S. D. Gimple, T. VandenBos, D. Friend, A. Alpert, D. Anderson, J. Jackson, J. M. Wignall, C. Smith, B. Gallis, J. E. Sims, D. Urdal, M. B. Windmer, D. Cosman, and L. S. Park. 1989. The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell* 59:335-3438.
31. Park, J. W., R. Stagg, G. D. Lewis, P. C. Carter, D. Maneval, D. J. Slamon, H. Jaffe, and H. M. Shepard. 1991. Anti-185HER2 monoclonal antibodies: biological properties and potential for immunotherapy, p. 193-211. In M. E. Lippman and R. B. Dickson (ed.), *Genes, oncogenes, and hormones: advances in molecular biology of breast cancer*. Kluwer, Boston.
32. Peles, E., S. S. Bacus, R. A. Koski, H. S. Lu, D. Wen, S. G. Ogden, R. B. Levy, and Y. Yarden. 1992. Isolation of the *neu*/HER2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 69:205-216.
33. Petch, L. A., J. Harris, V. W. Raymond, A. Blasband, D. C. Lee, and H. S. Earp. 1990. A truncated, secreted form of the epidermal growth factor receptor is encoded by an alternatively spliced transcript in normal rat tissue. *Mol. Cell. Biol.* 10:2973-2982.
34. Rubin, L. A., C. C. Kurman, M. E. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells *in vitro*. *J. Immunol.* 135:3172-3174.
35. Scott, G. K., J. M. Dodson, P. A. Montgomery, R. M. Johnson, J. C. Sarup, W. L. Wong, A. Ullrich, H. M. Shepard, and C. C. Benz. 1991. p185<sup>HER2</sup> signal transduction in breast cancer cells. *J. Biol. Chem.* 266:14300-14305.
36. Semba, K., N. Kamata, K. Toyoshima, and T. Yamamoto. 1985. A *v-erbB* related proto-oncogene, *c-erbB-2* is distinct from *c-erbB-1*/epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc. Natl. Acad. Sci. USA* 82:6497-6501.
37. Slamon, D., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 235:177-182.
38. Stancovski, I., E. Hurwitz, O. Leitner, A. Ullrich, Y. Yarden, and M. Sela. 1991. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB-2 receptor on tumor growth. *Proc. Natl. Acad. Sci. USA* 88:8691-8695.
39. Tal, M., C. R. King, M. H. Kraus, A. Ullrich, J. Schlessinger, and D. Givol. 1987. Human HER2 (*neu*) promoter: evidence for multiple mechanisms for transcriptional initiation. *Mol. Cell. Biol.* 7:2597-2601.
40. Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito, and K. Toyoshima. 1986. Similarity of protein encoded by the human *c-erbB-2* gene to epidermal growth factor receptor. *Nature (London)* 319:230-234.
41. Zabrecky, J. R., T. Lam, S. J. McKenzie, and W. Carney. 1991. The extracellular domain of p185/*neu* is released from the surface of human breast carcinoma cells, SK-Br-3. *J. Biol. Chem.* 266:1716-1720.